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(54) Title: METHODS FOR USING DENDRITIC CELLS TO ACTIVATE T CELLS			
(57) Abstract			
<p>This invention relates to methods of isolating and using human dendritic cells to present antigens for the induction of antigen-specific T cell-mediated immune responses. In particular, it relates to the isolation of dendritic cells from human blood, exposing the cells to native antigens or peptides, co-culturing the antigen-pulsed dendritic cells with T cells obtained from unprimed or weakly primed individuals for the stimulation of antigen-specific T cell proliferative and cytotoxic activities. The dendritic cell antigen presentation system described herein has a wide range of applications, including but not limited to, activation and expansion of large numbers of antigen-specific T cells for use in adoptive cellular immunotherapy against infectious diseases and cancer, use of antigen-pulsed dendritic cells as vaccines and/or immunotherapeutics, and an <i>in vitro</i> assay system for determining an individual's immune potential to any antigenic epitopes.</p>			

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METHODS FOR USING DENDRITIC
CELLS TO ACTIVATE T CELLS

1. INTRODUCTION

This invention relates to methods of isolating and using human dendritic cells to present antigens for the induction of antigen-specific T cell-mediated immune responses. In particular, it relates to the isolation of dendritic cells from human blood, exposing the cells to native antigens or peptides, co-culturing the antigen-pulsed dendritic cells with T cells obtained from unprimed or weakly primed individuals for the stimulation of antigen-specific T cell proliferative and cytotoxic activities. The dendritic cell antigen presentation system described herein has a wide range of applications, including but not limited to, activation and expansion of large numbers of antigen-specific T cells for use in adoptive cellular immunotherapy against infectious diseases and cancer, use of antigen-pulsed dendritic cells as vaccines and/or immunotherapeutics, and an in vitro assay system for determining an individual's immune potential to any antigenic epitopes.

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2. BACKGROUND OF THE INVENTION

2.1. GENERATION OF AN IMMUNE RESPONSE

The introduction of a foreign antigen into an individual elicits an immune response consisting of two major components, the cellular and humoral immune responses, mediated by two functionally distinct populations of lymphocytes known as T and B cells, respectively. A subset of T cells responds to antigen stimulation by producing lymphokines which "help" or activate various other cell types in the immune system. Another T cell subset is capable of developing into antigen-specific cytotoxic effector

cells, being able to directly kill antigen-positive target cells. On the other hand, the B cell response is primarily carried out by secretory proteins, 5 antibodies, which directly bind and neutralize antigens.

Helper T cells (Th) can be distinguished from classical cytotoxic T lymphocytes (CTL) and B cells by their cell surface expression of a 10 glycoprotein marker termed CD4. Although the mechanism by which CD4⁺ Th function has not been fully elucidated, the existence of functionally distinct subsets within the CD4⁺ T cell compartment has been reported (Mosmann and Coffman, 1989, Ann. Rev. 15 Immunol. 7:145-173). In the mouse, type 1 helper T cells (Th1) produce interleukin-2 (IL-2) and γ -interferon (γ -IFN) upon activation, while type 2 helper T cells (Th2) produce IL-4 and IL-5. Based on the profile of lymphokine production, Th1 appear to be 20 involved in promoting the activation and proliferation of other T cell subsets including CTL, whereas Th2 specifically regulate B cell proliferation and differentiation, antibody synthesis, and antibody class switching. Some CD4⁺ T cells, like CD8⁺ CTL, 25 appear to be capable of cytotoxic effector function.

A second T cell subpopulation is the classical CTL which express the CD8 surface marker. Unlike most Th, these cells display cytolytic activity upon direct contact with target cells, rather than 30 through the production of lymphokines. In vivo, CTL function is particularly important in situations where an antibody response alone is inadequate. There is a preponderance of experimental evidence that CTL rather than B cells and their antibody products play a 35 principal role in the defense against viral infections and cancer.

A salient feature of both T and B cell responses is their exquisite specificity for the

immunizing antigen; however, the mechanisms for antigen recognition differ between these two cell types. B cells recognize antigens by antibodies, either acting as cell surface receptors or as secreted proteins, which bind directly to antigens on a solid surface or in solution, whereas T cells only recognize antigens that have been processed or degraded into small fragments and presented on a solid phase such as the surface of antigen-presenting cells (APC). Additionally, antigenic fragments must be presented to T cells in association with major histocompatibility complex (MHC)-encoded class I or class II molecules. The MHC refers to a cluster of genes that encode proteins with diverse immunological functions. In man, the MHC is known as HLA. Class I gene products are found on all somatic cells, and they were originally discovered as targets of major transplantation rejection responses. Class II gene products are mostly expressed on cells of various hematopoietic lineages, and they are involved in cell-cell interactions in the immune system. Most importantly, MHC-encoded proteins have been shown to function as receptors for processed antigenic fragments on the surface of APC (Bjorkman et al., 1987, Nature 329: 506-512).

Another level of complexity in the interaction between a T cell and an antigenic fragment is that it occurs only if the MHC molecules involved are the same on the APC and the responding T cells. In other words, a T cell specific for a particular antigenic epitope expresses a receptor having low affinity for self MHC proteins, which when such MHC proteins on APC are occupied by the epitope, engage the T cell in a stronger interaction leading to antigen-specific T cell activation. The phenomenon of a T cell reacting with a processed antigen only when

presented by cells expressing a matching MHC is known as MHC-restriction.

The specificity of T cell immune responses for antigens is a function of the unique receptors expressed by these cells. The T cell receptor (TCR) is structurally homologous to an antibody; it is a heterodimer composed of disulfide-linked glycoproteins. Four TCR polypeptide chains known as α , β , γ , and δ have been identified, although the vast majority of functional T cells express the $\alpha\beta$ heterodimeric TCR. Transfer of α and β genes alone into recipient cells was shown to be both necessary and sufficient to confer antigen specificity and MHC-restriction (Dembic et al., 1986, Nature 320: 232-238). Thus, the $\alpha\beta$ TCR appears to be responsible for recognizing a combination of antigenic fragment and MHC determinants.

The apparent basis of MHC restriction is that CD4 $^{+}$ T cells express $\alpha\beta$ TCR which recognize antigenic fragments physically associated with MHC class II proteins, while the TCR on CD8 $^{+}$ CTL recognize MHC class I-associated fragments. Thus, CD4 $^{+}$ T cells can recognize only a restricted class of APC that are class II $^{+}$, whereas CD8 $^{+}$ CTL can interact with virtually any antigen-positive cells, since all cells express class I molecules. CD4 $^{+}$ CTL have been identified, and they are MHC class II restricted, and lyse target cells only if the latter express self-MHC class II determinants associated with specific antigenic fragments. Both CD4 and CD8 molecules also contribute to this interaction by binding to monotypic determinants on the MHC class II and I molecules, respectively.

A second type of TCR composed of $\gamma\delta$ heterodimers is expressed by a small percentage of T cells, but the involvement of $\gamma\delta$ T cells in antigen-

specific recognition is still poorly understood. Some studies have shown that functionally active $\gamma\delta$ T cells can be cytolytic in a MHC non-restricted manner.

5 In summary, the generation of an immune response begins with the sensitization of CD4⁺ and CD8⁺ T cell subsets through their interaction with APC that express MHC-class I or class II molecules associated with antigenic fragments. The sensitized 10 or primed CD4⁺ T cells produce lymphokines that participate in the activation of B cells as well as various T cell subsets. The sensitized CD8⁺ T cells increase in numbers in response to lymphokines and are capable of destroying any cells that express the 15 specific antigenic fragments associated with matching MHC-encoded class I molecules. For example, in the course of a viral infection, CTL eradicate virally-infected cells, thereby limiting the progression of virus spread and disease development.

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2.2. ANTIGEN PRESENTING CELLS

The presentation of antigens to T cells is carried out by specialized cell populations referred to as antigen presenting cells (APC). Typically, APC 25 include macrophages/monocytes, B cells, and bone marrow derived dendritic cells (DC). APC are capable of internalizing exogenous antigens, cleaving them into smaller fragments in enzyme-rich vesicles, and coupling the fragments to MHC-encoded products for 30 expression on the cell surface (Goldberg and Rock, 1992, Nature 357:375-379). Since APC express both MHC-encoded class I and class II glycoproteins, they can present antigenic fragments to both CD4⁺ and CD8⁺ T cells for the initiation of an immune response.

35 By definition, APC not only can present antigens to T cells with antigen-specific receptors, but can provide all the signals necessary for T cell

activation. Such signals are incompletely defined, but probably involve a variety of cell surface molecules as well as cytokines or growth factors.

5 Further, the factors necessary for the activation of naive or unprimed T cells may be different from those required for the re-activation of previously primed memory T cells. The ability of APC to both present antigens and deliver signals for T cell activation is

10 commonly referred to as an accessory cell function.

Although monocytes and B cells have been shown to be competent APC, their antigen presenting capacities in vitro appear to be limited to the re-activation of previously sensitized T cells. Hence, they are not

15 capable of directly activating functionally naive or unprimed T cell populations.

Although it had been known for a long time that APC process and present antigens to T cells, it was not shown until relatively recently that small

20 antigenic peptides could directly bind to MHC-encoded molecules (Babbit et al., 1985, Nature 317: 359; Townsend et al., 1986, Cell 44: 959). However, it is believed that normally, complex antigens are proteolytically processed into fragments inside the

25 APC, and become physically associated with the MHC-encoded proteins intracellularly prior to trafficking to the cell surface as complexes. Two distinct pathways for antigen presentation have been proposed (Braciale et al., 1987, Immunol. Rev. 98: 95-114). It was thought that exogenous antigens were taken up by

30 APC, processed and presented by the exogenous pathway to class II restricted CD4⁺ T cells, while the endogenous pathway processed intracellularly synthesized proteins, such as products of viral genes

35 in virally-infected cells, for association with MHC class I proteins and presentation to CD8⁺ CTL. However, although the two pathways in antigen processing and presentation may still be correct in

some respects, the distinction is blurred in light of recent findings that exogenously added antigens may also be presented to class I-restricted CTL (Moore et al., 1988, Cell 54: 777.)

The term "dendritic cells" refers to a diverse population of morphologically similar cell types found in a variety of lymphoid and non-lymphoid tissues (Steinman, 1991, Ann. Rev. Immunol. 9:271-296). These cells include lymphoid DC of the spleen, Langerhans cells of the epidermis, and veiled cells in the blood circulation. Although they are collectively classified as a group based on their morphology, high levels of surface MHC-class II expression, and absence of certain other surface markers expressed on T cells, B cells, monocytes, and natural killer cells, it is presently not known whether they derive from a common precursor or can all function as APC in the same manner. Further, since the vast majority of published reports have utilized DC isolated from the mouse spleen, results from these studies may not necessarily correlate with the function of DC obtained from other tissue types. (Inaba et al., 1987, J. Exp. Med. 166:182-194; Hengel et al., 1987 J. Immunol., 139:4196-4202; Kast et al., 1988, J. Immunol., 140:3186-3193; Romani et al., 1989, J. Exp. Med. 169:1169-1178; Macatonia et al., 1989, J. Exp. Med. 169:1255-1264; Inaba et al., 1990, J. Exp. Med. 172:631-6640). For example, despite high levels of MHC-class II expression, mouse epidermal Langerhans cells, unlike splenic DC, are not active APC in mixed leucocyte reaction (MLR), unless cultured with granulocyte-macrophage colony stimulating factor (GM-CSF) (Witmer-Pock et al., 1987, J. Exp. Med. 166:1484-1498; Heufler et al., 1988, J. Exp. Med. 167:700-705). Most human Langerhans cells express the CD1 and CD4 markers, while blood DC do not. Additionally, it has not been established the extent to which the

functional characteristics observed with mouse DC are applicable to human DC, especially the DC obtained from non-splenic tissues; in part, due to inherent 5 differences between the human and murine immune systems.

Recently, a few studies have described the isolation of human DC from the peripheral blood. (Young and Steinman, 1990, J. Exp. Med. 171:1315-1332; 10 Freudenthal and Steinman, 1990, Proc. Natl. Acad. Sci. USA 87:7698-7702; Macatonia et al., 1989, Immunol. 67:285-289; Markowicz and Engleman, 1990, J. Clin. Invest. 85:955-961). However, all reported isolation procedures invariably involve the use of sheep red 15 blood cells and/or fetal calf serum, which are potentially immunogenic foreign antigens that can be presented by DC to T cells, and if so, would interfere with the antigen-specific responses desired. Most importantly, it has not been determined prior to 20 Applicants' invention whether human DC can, in fact, present exogenous antigens to naive T cells because human DC have only been tested as stimulators for T cell reactivity in MLR. Human DC which are active in MLR have not been shown to be capable of presenting 25 exogenous antigens for primary or secondary T cell activation.

3. SUMMARY OF THE INVENTION

30 The present invention relates to the isolation of human DC from the peripheral blood, their use as antigen presenting cells for the activation of T cell responses, and an in vitro method for assessing immune responsiveness of both unprimed and primed 35 individuals to potentially immunogenic epitopes using dendritic cells as APC, and CD4⁺ and/or CD8⁺ T cells as responders. Because DC are present at extremely low quantities in the human peripheral blood, their

enrichment and purification are necessary in order to obtain adequate numbers for pulsing with antigens for the induction of both primary and secondary helper and 5 cytotoxic T cell responses in vitro.

The invention is based, in part, on Applicants' discovery that DC partially purified from human blood by sequential density gradient centrifugation function as potent APC for the 10 sensitization of autologous naïve T cells. As shown in the working examples described herein in Example 7, *infra*, DC exposed to keyhole limpet hemocyanin (KLH) and sperm whale myoglobin (SWM) in vitro stimulate primary antigen-specific CD4⁺ Th proliferative 15 responses, while similarly prepared autologous monocytes are not effective. In vitro primed KLH-specific CD4⁺ T cell lines can be expanded long-term; i.e., at least several months in the presence of low doses of interleukin-2 (IL-2) and/or interleukin-4 20 (IL-4) plus APC and antigen, with retention of both antigen-specificity and biologic activity.

Additionally, KLH-specific CD8⁺ cytotoxic T cells have been generated using a similar procedure in which DC pulsed with KLH are used to sensitize 25 purified CD8⁺ T cells in the absence of detectable CD4⁺ cells. When DC are used to present a synthetic peptide derived from the human immunodeficiency virus (HIV) gag antigen to autologous T cells obtained from HIV seronegative donors, i.e., individuals who are not 30 infected with HIV and have never been exposed to HIV or HIV associated antigens, HIV-specific cytotoxic T cell lines have been established.

A wide variety of uses for this antigen presentation system is encompassed by the invention 35 described herein, including but not limited to, the activation and expansion of antigen-specific T cells in vitro for use in adoptive cellular immunotherapy of

infectious diseases and cancer, the in vivo administration of antigen-pulsed DC as vaccines for priming primary responses or for re-activating 5 secondary immune responses, and the identification of antigenic epitopes for vaccine development.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Illustration of the procedures for 10 isolating purified DC from human blood.

FIG. 2. Cytofluorographic analysis of DC stained with monoclonal antibodies after the first Nycodenz/Nycoprep centrifugation.

15 FIG. 3. Cytofluorographic analysis of DC stained with monoclonal antibodies after the second Nycodenz/Nycoprep centrifugation.

20 FIG. 4. Cytofluorographic analysis of DC stained with monoclonal antibodies after Nycodenz/Nycoprep centrifugation followed by antibody panning.

25 FIG. 5. Generation of primary T cell proliferative responses to antigens presented by autologous dendritic cells in vitro. CD4⁺ T cells and their subsets were obtained from normal human peripheral blood and cultured with autologous DC or monocytes pulsed with KLH, SWM, or no antigen for seven days. T cell proliferation was assessed by [³H]-thymidine incorporation assay.

30 35 FIG. 6. Generation of secondary T cell proliferative responses to an antigen presented by autologous dendritic cells in vitro. CD4⁺ T cells were obtained from normal human

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peripheral blood and cultured with autologous DC pulsed with KLH or SWM. The sensitized T cells were expanded in culture by periodic restimulation with antigen-pulsed monocytes, and a combination of lymphokines, IL-2 and IL-4. After 6-8 weeks, the resultant T cells were analyzed for their proliferative response to KLH or SWM presented by autologous monocytes in a [³H]-thymidine incorporation assay.

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FIG. 7. Comparison between dendritic cells and monocytes for their ability to stimulate secondary antigen-specific CD4⁺ T cell proliferative responses. CD4⁺ T cells were obtained from normal human peripheral blood and cultured with autologous DC or monocytes in the presence of KLH, SWM or tetanus toxoid.

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FIG. 8. Generation of antigen-specific cytotoxic T cell responses to an antigen presented by autologous dendritic cells in vitro. CD8⁺ T cells were obtained from normal human peripheral blood and cultured for 10 days with autologous DC which had been pulsed with KLH in the presence of hypertonic sucrose. The sensitized T cells were expanded in culture by low doses of IL-2 and/or IL-4, and periodic restimulation with autologous monocytes pulsed with KLH every eight to ten days. After six to eight weeks, the resultant T cells were analyzed for their cytotoxic activity in a ⁵¹Cr release assay. Target cells were ⁵¹Cr

labelled autologous monocytes pulsed with or without KLH.

5 Fig. 9. Generation of primary HIV-peptide specific cytotoxic T cell responses by in vitro stimulation with autologous dendritic cells pulsed with an HIV gag peptide 'b', in the presence of IL-1 and IL-2 for seven days.

10 The sensitized T cells were expanded by periodic restimulation with autologous monocytes exposed to HIV gag peptide b every eight to ten days, and a combination of lymphokines, IL-2 and IL-4. After six to

15 eight weeks, the resultant T cells were analyzed for their cytotoxic activity in a ⁵¹Cr release assay. Target cells were ⁵¹Cr labelled autologous monocytes pulsed with or without HIV peptide 'a' or 'b'. Specific

20 cytolytic activity was blocked by monoclonal antibodies directed to CD8 or HLA Class I antigens.

5. DETAILED DESCRIPTION OF THE INVENTION

25 The present invention relates to methods of isolating and using dendritic cells for activating antigen-specific T cell responses. Although the specific procedures and methods described herein are exemplified using DC isolated from human blood, they

30 are merely illustrative for the practice of the invention. Analogous procedures and techniques are equally applicable.

Therefore, DC may be isolated using variants of the procedure described herein, pulsed with any antigens or fragments thereof, and incubated with primed or unprimed T cells and their subsets. The scope of this invention encompasses the use of antigen-pulsed DC as APC both in vitro and in vivo.

5.1. ISOLATION OF HUMAN BLOOD DENDRITIC CELLS

The present invention relates to an antigen presentation system using DC for the activation of T cells in vitro and in vivo. Due to their presence in low numbers in most tissues, DC must first be isolated and enriched. Although DC are found in both lymphoid and non-lymphoid tissues, a natural and easily accessible source of DC in man is the peripheral blood, which contains an estimate of fewer than 1 DC per 100 white blood cells.

The potency of the accessory cell function of DC in antigen presentation allows for the use of these cells in relatively small numbers when enriched, and absolute purity is not necessary for the generation of a T cell priming effect in vitro. For the in vitro activation of T cells, APC containing >30% DC are generally adequate. However, it is most preferable that a highly purified DC population (about 90%) be obtained for use in in vivo priming, for immunization in animals for the generation of monoclonal antibodies to DC-specific markers, and for the preparation of cDNA libraries in an attempt to identify novel cytokine genes.

Human DC may be isolated from any tissues where they reside, using a variety of separation methods. Example 6, infra, presents variants of such methods as illustrations for isolating DC from the human peripheral blood. This procedure is principally designed to avoid the exposure of DC to antigens such as fetal calf serum, sheep red blood cells and xenogeneic monoclonal antibodies, which have been used routinely in the separation of peripheral blood mononuclear leucocytes (PBML). Since DC may be able to present such xenogeneic antigens to naive T cells, even in the absence of other exogenously added antigens, conventional methods of DC isolation may lead to activation of T cells not specific for the

antigens of interest, thus potentially masking the responses sought. In accordance with this aspect of the invention, human PBML may be isolated from blood samples, particularly buffy coats or leucocytes prepared by apheresis, by Ficoll Hypaque gradient centrifugation followed by Percoll density centrifugation. The high buoyant density (HD) fraction contains T cells, B cells, and DC, whereas monocytes are in the low buoyant density (LD) fraction. The HD fraction can then be subjected to centrifugation in Nycodenz/Nycoprep which separates DC which are in the LD fraction from the HD fraction which contains T and B cells. At this point, DC may be further enriched using additional protocols, depending on the level of purity required. For use in *in vitro* activation of T cells, DC obtained at this stage ($\geq 30\%$ DC) can be pulsed immediately with any antigen of interest.

Alternatively, DC may be isolated by procedures involving repetitive density gradient centrifugation, positive selection, negative selection, or a combination thereof. For example, the LD Nycodenz fraction described above may be subjected to a second round of Nycodenz/Nycoprep centrifugation. The LD fraction contains a highly purified DC population of 80-90%. The LD fraction after the first Nycodenz/Nycoprep step may be negatively selected by panning using antibodies to remove non-DC to give rise to a 80-90% DC preparation. However, this step involves monoclonal antibodies which are potential foreign antigens, thus it is a less preferable approach. Positive selection methods may utilize affinity chromatography with antibodies directed to DC cell surface markers. Since a human DC-specific antibody is not currently available, positive selection does not necessarily require the use of antibodies that recognize DC-specific determinants.

For example, B cells and monocytes may be depleted first from the DC-containing fraction after density gradient centrifugation, plastic adhesion, and Fc receptor panning, then an antibody to MHC-Class II antigen can be used to positively select for DC. Negative selection includes modifications of the protocol disclosed herein, *supra*. In essence, a DC-containing cell preparation may be reacted with one or more antibodies directed at cell surface antigens not expressed by DC for the removal of non-DC. Antibodies to any T cell, B cell, monocyte, and granulocyte markers may be used. Examples of such antibodies include anti-CD3, anti-CD4, anti-CD5, and anti-CD8 specific for T cells; anti-CD12, anti-CD19 and anti-CD20 specific for B cells; anti-CD14 specific for monocytes; and anti-CD16, and anti-CD56 specific for natural killer cells. These antibodies may be applied in any combination repeatedly or in a sequential manner for the enrichment of DC. Upon binding to the antibodies, the cells may be removed by adsorption to a solid surface coated with an anti-mouse antibody column, as the majority of monoclonal antibodies directed at cell surface markers are of mouse origin, or if the antibodies are conjugated with biotin, the antibody-bound cells can be removed by an avidin-coated surface; or if the antibodies are conjugated to magnetic beads, the cells expressing antigens recognized by the antibodies can be removed in a magnetic field.

5.2. USE OF DENDRITIC CELLS AS ANTIGEN PRESENTING CELLS

The initiation of an immune response is mediated by antigen presenting cells, which process complex antigens into smaller fragments by enzymatic degradation, and present them in association with MHC-encoded molecules to T cells. Although

macrophages/monocytes have been studied most extensively as APC, murine DC have been shown to also possess accessory cell function. The present invention demonstrates that DC isolated from human blood present antigens for the activation of antigen-specific CD4⁺ and/or CD8⁺ T cells in settings where monocytes cannot.

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**5.2.1. ANTIGENIC SYSTEMS FOR PRESENTATION
BY DENDRITIC CELLS**

The potent accessory cell function of DC provides for an antigen presentation system for virtually any antigenic epitopes which T and B cells are capable of recognizing through their specific receptors. Example 7, *infra*, demonstrates that human DC can present both complex protein antigens and small peptides to CD4⁺ T cells as well to as CD8⁺ CTL. T cell activation is manifested by T cell proliferation and/or cytotoxicity in response to antigen. Hence, DC may be useful in presenting antigens encoded by infectious agents such as viruses and microorganisms as well as tumor antigens expressed by cancer cells (Urban and Schreiber, 1992, Ann. Rev. Immunol. 10: 617-644).

Infectious agents against which the present invention may be applicable in the induction of an immune response include, but are not limited to, bacteria, parasites, fungi, and viruses. The multitudes of antigens encoded by these agents, which may be processed and presented by DC include but not limited to, external surface proteins, and structural proteins including internal enzymes. For example, antigens encoded by any genes of the HIV genome including the env, gag, pol, nef, vif, rev, and tat genes may all be presented by DC to T cells. In addition, a variety of other infectious agents including hepatitis B virus, hepatitis C virus, cytomegalovirus, herpes simplex virus, varicella zoster, and *Mycobacterium* species are encompassed within the scope of the invention.

A large number of human tumor-associated antigens have been identified by monoclonal antibodies (Reisfeld and Cheresh, 1987, Adv. Immunol. 40: 323-377). Although these cellular antigens are

selectively expressed in higher quantities by certain tumor cells, it has not been established that they naturally elicit an immune response in cancer patients or can be used effectively to induce such a response.

5 Progress in this area is, in part, hampered by the lack of an adequate in vitro system for analyzing human anti-tumor immune responses, particularly T cell-mediated responses. Unlike animal tumor models

10 in which tumor-reactive T and B cells can be induced through hyperimmunization with tumor cells or tumor antigens, human tumor cells or oncogenic proteins may not be injected into humans for stimulating tumor-reactive T cells due to ethical limitations. Thus,

15 most human studies have utilized lymphocytes obtained from cancer patients whose cells presumably have been exposed to antigens expressed by their autologous tumor cells in vivo. However, it has been shown in some systems that tumor development is accompanied by

20 a down-regulation of tumor specific immune responsiveness mediated by suppressor cells, and if so, T cells isolated from cancer patients may have already come under the influence of such suppression in vivo so as to not function in a manner similar to

25 that of T cells obtained from tumor-immune hosts. Moreover, these attempts to activate human tumor-reactive T cells have generally used monocytes as APC, which are shown herein to be much less effective APC than DC, especially if the T cells have not been

30 primed adequately in vivo against the tumor antigens. Alternatively, cytotoxic lymphocytes have been directly activated by use of high doses of lymphokines such as IL-2, but this approach suffers from a lack of tumor specificity and various toxic side effects.

35 The DC described herein establish an ideal system for assessing and stimulating human anti-tumor responses, using naive lymphocytes from normal, presumably un suppressed individuals or T cells from

tumor-bearing patients. The potent accessory cell function of DC may be able to present tumor antigens to T cells from cancer patients, whose immune response 5 is apparently inadequate to eliminate the tumors *in vivo*. The activated T cells can be expanded *in vitro* for use in adoptive immunotherapy. Whole tumor cells in viable or irradiated form, tumor membrane preparations, and tumor antigens purified from natural 10 sources or expressed as recombinant products may be used to pulse DC for presentation to autologous T cells.

Recently, oncogene products have been shown to be capable of inducing murine T cell activities. 15 For example, oncogenic forms of the ras gene product p21, and the fusion product p210 of the bcr-abl gene induce T cell proliferative responses, when used to immunize mice (Peace et al., 1991, J. Immunol. 146: 2059-2065; Chen et al., 1992, Proc. Natl. Acad. Sci. USA 89: 1468-1472). Thus, oncogenic proteins which 20 are different from their normal cellular counterparts as a result of amino acid substitutions may possess new immunogenic determinants that are recognizable by T cells. It is not necessary that such proteins be 25 expressed naturally on the cell surface, as cytoplasmic and nuclear proteins may be processed, attached to MHC-encoded products intracellularly, and translocated to the cell surface in a complex form (Gould et al., 1989, J. Exp. Med. 170: 1051-1056). 30 Since oncogene products are expressed in a variety of tumor types including colon cancer, leukemia and lymphoma, DC may be used to activate T cells against such cancers. Human T cells, particularly CTL specific for oncogene products, may be induced by DC presentation, and expanded by procedures similar to 35 that described herein for the procurement of large numbers of tumor-specific T cells for adoptive cellular immunotherapy *in vivo*.

Bacterial, parasitic, fungal, viral, and tumor antigens of cellular or viral origin may be introduced to DC by addition to DC cultures, by the 5 osmotic lysis of pinosomes after pinocytotic uptake (Moore et al., 1988, Cell 54: 777-785), or by uptake in antigen containing liposomes. Antigens may be used as purified naturally occurring whole polypeptides, purified recombinant whole polypeptides, whole 10 organisms or cells in viable or dead forms, protein fragments generated by enzymatic digestion, or synthetic peptides produced by solid phase chemical method (Creighton, 1983, Protein Structures and Molecular Principles, W.H. Freeman and Co., N.Y. pp 15 50-60). The amount of antigens necessary for pulsing DC may vary depending on the nature, size, and purity of the molecules. In general, polypeptides may be used at 1-100 µg/ml, and small peptides at 1-50 µg/ml. Introduction by osmotic lysis of pinosomes requires 20 larger amounts of proteins in the range of 200-500 µg/10⁶ APC. Alternatively, exogenous genes encoding specific antigens of interest or expression vectors containing such genes or portions thereof may be incorporated into DC in expression vectors using 25 conventional methods, including transfection, recombinant vaccinia viruses and retroviruses. This approach causes the continual expression of integrated genes, leading to MHC occupancy by the gene products. Any of the above-mentioned methods for introducing 30 exogenous antigens into DC as well as any others commonly used by those skilled in the art are hereinafter collectively referred to as pulsing of APC. Antigen pulsing of DC may occur prior to co-culture with T cells or antigens may be added to 35 cultures containing both DC and T cells at the same time.

5.2.2. INDUCTION OF PRIMARY AND SECONDARY

T CELL RESPONSES IN VITRO

One of the most outstanding characteristics of DC function is their ability to present antigens for the induction of primary T cell responses. Since none of the studies performed in this area have utilized human DC, it has not been established that human DC can present exogenous antigens to activate unprimed T cells. In a specific embodiment by way of example, described in Example 7, *infra*, naive T cells isolated from individuals not previously exposed to an antigen can be primed *in vitro* by DC pulsed with that antigen. Antigen-pulsed DC activate both CD4⁺ T cell proliferative responses and CD8⁺ CTL responses. This is in contrast to monocytes which are only competent antigen presenters *in vitro* to already primed T cells for secondary responses.

For the induction of a primary T cell response *in vitro*, DC may be used immediately after antigen pulsing or they may be maintained in the presence of GM-CSF and/or other cytokines prior to antigen pulsing and co-culture with T cells (Markowicz and Engleman, 1990, *J. Clin. Invest.* 85:951). It is known that although DC may process antigens for only a short time period *in vitro*, they retain the antigenic fragments bound to MHC molecules for a significant time period, and thus, may be used even several days after antigen pulsing (Inaba et al., 1990, *J. Exp. Med.* 172: 631-640).

In order to augment the magnitude of the priming effects of DC, exogenous lymphokines and monokines may be added to the cultures, including but not limited to, IL-1 and IL-2, at 0.1-100 U/ml. Higher concentrations of such cytokines may also be used; however, they may induce antigen non-specific T cell activities. It is not required that any exogenous factors be present, since DC appear to produce all the necessary signals for T cell

activation. However, for the long-term expansion of T cells after DC priming, lymphokines such as IL-2 and IL-4 at 1-100 U/ml may be used to greatly facilitate the rate at which T cells propagate.

T cells and their subsets may be obtained from various lymphoid tissues for use as responder cells. Such tissues include but are not limited to, spleens, lymph nodes, and peripheral blood. The cells may be co-cultured with antigen-pulsed DC as a mixed population or as a purified subset, depending on the type of response and/or the composition of the stimulated cells desired. For example, in a culture designed to generate HIV-specific T cells, CD4⁺ T cells may be depleted prior to culture since they are susceptible to HIV infection. Thus, it may be more desirable to culture purified CD8⁺ T cells with DC pulsed with HIV antigens to generate HIV-specific CTL. This is particularly important if T cells are obtained from HIV-infected patients for restimulation and expansion *in vitro*, the depletion of CD4⁺ T cells reduces the likelihood of HIV contamination of cultures. In addition, early elimination of CD4⁺ cells prevents the overgrowth of CD4⁺ cells in a mixed culture of both CD4⁺ and CD8⁺ T cells over time. It is demonstrated in Example 7.2.2, *infra*, that antigen-specific CTL can be induced in the absence of detectable CD4⁺ T cells, when stimulated with antigen-pulsed DC. T cell purification may be achieved by positive, or negative selection, including but not limited to, the use of antibodies directed to CD2, CD3, CD4, CD5, and CD8.

T cells may be isolated from an individual not previously exposed to a particular antigen.

Antigen-pulsed DC may be used also to re-activate previously primed T cells for a secondary response, in which case, the donors may be tested first for prior

antigen exposure by the presence of serum antibodies or a detectable T cell response. Antigen-pulsed DC not only sensitize naive T cells but they also
5 stimulate a stronger secondary T cell response in vitro than monocytes can.

Once naive T cells have been activated by DC, they may be restimulated by any APC including autologous DC, autologous normal or Epstein Barr
10 virus-transformed B cells, or monocytes, and expanded with lymphokines. The expanded T cells may be administered alone into an individual, or in combination with lymphokines such as IL-2 and/or IL-4, by repeated injections or continuous infusion via any
15 conventional route.

The use of DC to activate T cells depends on a number of conditions. For example, patients with late stage HIV infection may not be able to generate competent anti-viral T cell responses; and thus, CTL
20 from healthy HLA-matched individuals such as siblings, may be primed with HIV antigen-pulsed DC in vitro, expanded in numbers, and administered into the patients. The effects of therapy can be monitored on the basis of changes in viral load, the number of CD4⁺
25 T cells in the patients' blood, and clinical course. On the other hand, HIV-infected patients with early-stage disease may still possess T cells capable of becoming HIV-specific CTL. In this case, the proposed treatment may involve ex vivo re-activation of their
30 own T cells by autologous or HLA-matched homologous antigen-pulsed DC followed by reinfusion of their activated T cells. A similar approach may be applicable in other viral infections and in cancer patients, depending on the stage of the disease, the
35 availability of HLA-matched donor cells, and the ability of the patients' own T cells to mount a competent antigen-specific immune response.

5.2.3. INDUCTION OF PRIMARY AND SECONDARY
T CELL RESPONSES IN VIVO

The ability of DC to process and retain antigenic fragments for several days permits their use as potent immunogens in vivo. DC may be pulsed with antigens according to the various methods described in Section 5.2.1, supra, washed, and administered in vivo as vaccines and/or immunotherapeutics for the elicitation of an immune response or augmentation of a pre-existing but weak response. It is possible that immunization with antigen-pulsed DC can increase both the magnitude and the specificity of a response. It may be desirable to repeat such immunizations at time intervals of days or weeks. The potency of DC as APC may alleviate the need of using conventional adjuvants to augment the response, although it does not preclude the use of adjuvants to further enhance immune reactivity. Antigen-pulsed DC may be used to prime and/or boost immune responses in vivo against infectious agents and cancer.

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**5.2.4. A METHOD FOR IDENTIFICATION
OF IMMUNOGENIC PEPTIDES**

Currently, the conventional methods for assessing immunogenicity of proteins involve the immunization of animals with the proteins or fragments thereof, and subsequently testing for their secondary T cell or antibody responses in vitro or in vivo. The requirement of an in vivo priming step is both labor-intensive and time-consuming. The ability of DC to induce primary T cell responses in vitro alleviates the need for in vivo animal priming. T cells may be obtained from any individuals with or without previous antigen exposure and tested for their recognition of defined epitopes presented by autologous DC.

The DC antigen presentation system involves the culturing of T cells or their subsets with autologous or HLA-matched homologous DC in the presence of any antigen. Antigens may be introduced through gene transfer using infectious viral vectors or used in recombinant form or purified from natural sources, in whole or in part. Both T cell proliferative and cytotoxic activities can be measured. This system provides for a rapid method for analyzing and mapping T cell reactivities with various antigenic epitopes by any individuals, thereby facilitating the design of "tailor-made" vaccines based on each individual's own immune repertoire and pattern of T cell recognition. It further allows the comparison of the magnitude of T cell responses to different epitopes, thereby identifying immunodominant epitopes for CD4⁺ and/or CD8⁺ T cells for the induction of the strongest immune response.

5.2.5. A SOURCE FOR IDENTIFICATION
OF NOVEL CYTOKINES

5 The ability of antigen-pulsed DC to activate
T cell responses indicates that DC produce secondary
signals to T cells, in addition to engaging the T cell
receptors by MHC-antigen complexes. Such additional
signals may be novel cytokines or membrane-bound
accessory or adhesion molecules involved in cell-cell
10 contact. Therefore, DC may be used as a source for
identifying novel T cell activation or accessory
molecules and their genes.

15 In order to identify DC-derived molecules,
including novel cytokines, DC may be established first
as long-term cultured cell lines such as using GM-CSF
or transformed into immortal cell lines by tumor
viruses. DC culture supernatants may be assayed for
their ability to activate T cells in proliferative and
cytotoxicity assays. For example, a biologic assay
20 may be designed in which the TCR is triggered in the
absence of APC. This can be achieved by incubating
isolated T cells with an anti-CD3 antibody anchored on
a solid surface. T cell activity is then determined
in the presence or absence of DC culture supernatants.
25 Any biologic activity in the supernatants that
enhances T cell activation may be further defined by
conventional biochemical methods such as SDS-
polyacrylamide gel electrophoresis, high performance
liquid chromatography, and amino acid sequence
30 determination. The coding sequences of those
molecules may be molecularly cloned by conventional
recombinant DNA technology.

6. EXAMPLE: ISOLATION AND PURIFICATION
OF HUMAN DENDRITIC CELLS

6.1. MATERIALS AND METHODS

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6.1.1. CELL SEPARATION

Human DC were obtained from buffy coats of healthy, HIV-1 seronegative donors. Peripheral blood mononuclear leucocytes (PBML) were isolated by Ficoll-Hypaque gradient centrifugation (Boyum, 1968, Scand. J. Clin. Lab. Invest: 21:21-29). Blood dendritic cells (DC) were further separated by the methods described in FIG. 1. In brief, PBML were separated into LD and HD fractions in a four-step discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) (Markowicz and Engleman, 1990, J. Clin. Invest. 85:955-961). The HD fraction containing DC was collected and cultured in culture media in Teflon vessels for 16-18 hours at 37°C. Thereafter, the cells were centrifuged over a Nycoenz/Nycoprep discontinuous gradient (Nycomed Pharma AS, Oslo, Norway). DC were contained entirely in the LD fraction, occupying 30-40% of the whole population. This partially purified DC population was used for all the T cell priming and activation experiments described in Example 7, *infra*.

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This enriched DC population could be further purified by another round of Nycoenz/Nycoprep centrifugation, and the LD fraction obtained thereby contained 80-90% DC. Alternatively, the LD fraction after the first Nycoenz/Nycoprep step was incubated with antibody-coated petri dishes to remove CD3+, CD14+, CD16+, and CD20+ cells. The non-adherent cell population contained 80-90% DC. Both procedures consistently produced a yield of 1-2.5X10⁶ cells from about 400-500 ml of whole blood.

The purity of DC following each step of DC enrichment was assessed by staining with an anti-HLA-

DR (anti-MHC class II) antibody (CA141) conjugated to fluorescein, and phycoerythrin-conjugated anti-CD14 (anti-monocyte). Cytofluorographic analysis of the entire cell population was assessed by Fluorescence Activated Cell Sorter. HLA-DR⁺ but CD14⁻ cells represented the DC population.

T cell subsets were obtained from the high density Nycodenz fraction followed by an antibody panning technique using monoclonal antibodies directed to T cell markers. Based on cytofluorographic analysis, >95% of the cells expressed the antigen for which they were selected.

6.2. EXAMPLES

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6.2.1. ISOLATION OF HIGHLY PURIFIED PERIPHERAL BLOOD DENDRITIC CELLS

Highly purified DC have been obtained using a combination of density gradient centrifugation, and antibody panning procedures. The purity of DC was monitored using a monoclonal antibody specific for HLA-DR (class II) antigens, since DC specific antibodies were not available. DC can be readily distinguished from other PBML on the basis of their high level expression of MHC-class II determinants and their concurrent lack of CD14 expression, which is associated with monocytes. The brightly staining MHC-class II⁺ DC were also negative for a variety of known T, B, and NK cell markers.

At the end of the first Nycodenz/Nycoprep centrifugation step, the LD fraction contained 30-40% DC (FIG. 2). The DC fraction could be further enriched by a second Nycodenz/Nycoprep centrifugation (FIG. 3), or by panning with monoclonal antibodies specific for non-DC markers (FIG. 4). Both procedures gave rise to a highly purified population of 80-90% DC.

For the purpose of antigen pulsing experiments described herein in Example 7, *infra*, only partially purified DC were necessary. These enriched 5 DC were isolated in the LD fraction after the first Nycomedenz/Nycoprep gradient. The preparation always contained $\geq 30\%$ DC which were then used for antigen pulsing.

10 7. EXAMPLE: USE OF HUMAN DENDRITIC CELLS FOR
ACTIVATING ANTIGEN-SPECIFIC T CELL RESPONSES

7.1. MATERIALS AND METHODS

15 7.1.1. ANTIGENS AND REAGENTS

Purified sperm whale myoglobin (SWM) and keyhole limpet hemocyanin (KLH), (Sigma, St. Louis, MO) were used as antigens. Tetanus toxoid was purchased from Michigan Department of Public Health. HIV gag peptide antigens, 'a' 418-433 20 (KEGHQMKDCTERQANP) and 'b' 265-279 (KRWIILGLNKIVRMYC) were synthesized on an automated peptide synthesizer and their purity was assessed by HPLC and amino acid analysis. Based on previous studies, peptide 'a' has been reported to be recognized by HLA-A2 restricted, 25 HIV-specific cytotoxic T cell lines (Claverie et al., 1988, Eur. J. Immunol. 18: 1547-1553) and peptide 'b' is recognized by cytotoxic T cells in association with HLA-B27 (Nixon et al., 1988, Nature 336: 484-487).

30 7.1.2. ANTIGEN PULSING OF DENDRITIC CELLS

For the induction of a CD4 $^{+}$ T cell-mediated proliferative response, KLH, SWM, and tetanus toxoid were added to cultures containing DC and CD4 $^{+}$ T cells. For the induction of CD8 $^{+}$ CTL responses, KLH was 35 introduced to DC or monocytes at a concentration of 250-500 μ g/10 6 APC by the osmotic lysis of pinosomes (Moore et al. 1988, Cell 54, 777-785). For pulsing

with HIV gag peptides, DC or monocytes were incubated with 5 µg/ml of the peptides for 2 hours before addition to the culture. Alternatively, HIV peptides 5 could be added directly to co-cultures containing both DC and T cells.

7.1.3. PRIMARY AND LONG-TERM T-CELL CULTURES

Purified human CD4⁺, and CD8⁺ T cell subsets 10 at 1 X 10⁵/well were cultured in microliter wells. The incubation medium was RPMI 1640 medium supplemented with 10% heat inactivated human serum, 2mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. The cells were stimulated with autologous 15 dendritic cells or monocytes at 1 X 10⁴/well pulsed in vitro with SWM, KLH, tetanus toxoid or HIV gag peptides. For the generation of cytotoxic T cells, the primary cultures also contained IL-1 at 2.5 U/ml, and IL-2 at 0.5 U/ml. T cells were maintained in 20 culture with a combination of lymphokines; i.e., IL-2 at 1-2 U/ml, and IL-4 at 1-3 U/ml. Every week, these cells were restimulated with autologous monocytes exposed in vitro to the specific antigens originally used for priming.

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7.1.4. PROLIFERATION ASSAY

Enriched CD4⁺ T cells from normal human peripheral blood were cultured with autologous DC or 30 monocytes in the presence or absence of antigens for 7 days. The proliferative response was assessed by [³H]-thymidine incorporation.

7.1.5. CYTOTOXICITY ASSAY

After approximately 6-8 weeks, the resultant 35 CD8⁺ T cells were analyzed for cytotoxicity activity in a standard ⁵¹Cr release assay. Target cells were ⁵¹Cr labelled autologous monocytes which were either

untreated or pulsed with KLH, SWM, or HIV gag peptides. In antibody blocking experiments, the different MAbs were added at 20 µg/ml to the cultures 5 in the ³Cr release assay. Spontaneous ³Cr release from target cells in the absence of CTL was <15%. Percentage specific ³Cr release from lysed target cells was calculated as:

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$$\frac{100 \times (\text{cpm (sample release)} - \text{cpm (spontaneous release)})}{(\text{cpm (total release)} - \text{cpm (spontaneous release)})}$$

7.2. EXAMPLES

7.2.1. GENERATION OF ANTIGEN-SPECIFIC CD4⁺ T CELL PROLIFERATIVE RESPONSES IN VITRO USING DENDRITIC CELLS

KLH, SWM,, and HIV were chosen as antigens because the vast majority of humans have never been sensitized to these antigens *in vivo*, and their T 20 cells do not mount proliferative or cytotoxic responses to the antigens *in vitro*, when macrophages/monocytes are used as APC.

In order to test the ability of human DC to present such exogenous antigens, DC were isolated from 25 human blood, co-cultured with an enriched fraction of autologous CD4⁺ T cells in the presence of KLH or SWM, and a primary CD4⁺ T cell-mediated proliferative response was induced (FIG. 5). As a control, similarly prepared monocytes did not induce a 30 response. In addition, the CD4⁺ T cells were further subdivided into naive and memory T cells fractions by an antibody designated UCHL-1. The UCHL-1⁺ and UCHL-1⁻ T cells express two isoforms of the CD45 molecule, CD45RO and CD45RA, which correspond to memory and 35 naive T cells, respectively. When the two subsets were incubated with antigen-pulsed DC, it was the CD4⁺ UCHL-1⁻ T cells that proliferated, providing further

evidence that the observed response was mediated by previously unprimed naive T cells.

The in vitro primed CD4⁺ T cells specific for KLH or SWM were maintained in culture by repetitive stimulation with autologous antigen-pulsed monocytes, and expanded in numbers by a combination of IL-2 and IL-4 for several weeks. When the cultured T cells were again incubated with antigen-pulsed monocytes, a strong secondary antigen-specific proliferative response was demonstrated (FIG. 6).

When DC and monocytes were compared for their ability to present antigens for re-activating secondary T cell responses, it was observed that DC were capable of stimulating a stronger response than monocytes could (FIG. 7). Since most individuals have been exposed to tetanus toxoid as a result of vaccination, it was presumed that the anti-tetanus toxoid response was a secondary response mediated by previously primed T cells, as supported by the finding that even monocytes were able to stimulate a weak yet detectable CD4⁺ proliferative response. As a control, monocytes could not present KLH or SWM to unprimed T cells.

In conclusion, DC are capable of processing and presenting whole native protein antigens to CD4⁺ T cells in inducing a primary antigen-specific proliferative response in vitro. It is noteworthy that both primary and secondary T cell responses can be induced by antigen-pulsed DC, indicating that DC are uniquely able to both prime naive T cells from unimmunized normal individuals and re-activate previously primed T cells, while monocytes are not. Further, it is the CD4⁺ UCHL-1⁺ naive T cell population that is primarily responsible for the antigen-specific proliferative response. The in vitro priming effect of DC does not require the addition of exogenous lymphokines, indicating that DC produce all of the

necessary signals in antigen presentation leading to the activation of T cells.

5 7.2.2. GENERATION OF ANTIGEN-SPECIFIC CD8⁺ CYTOTOXIC T CELLS *IN VITRO* USING DENDRITIC CELLS

In addition to priming CD4⁺ helper T cells to exogenous antigens, DC were shown to be capable of activating MHC class I-restricted antigen-specific cytotoxic T cells. DC were pulsed with KLH and cultured with purified autologous CD8⁺ T cells in the presence of low doses of IL-1 and IL-2. The T cells were maintained by periodic restimulation with autologous monocytes pulsed with KLH, and expanded by IL-2 and IL-4 for 6-8 weeks. The resultant CD8⁺ T cell lines were able to kill autologous target cells pulsed with KLH, while untreated target cells were not lysed. (FIG. 8).

As DC have been shown to display potent antigen-presenting function for presenting complex protein antigens such as KLH, they were further examined for their ability to directly activate CTL specific for antigens associated with infectious agents, using HIV peptides as an example. DC from healthy HIV seronegative donors were exposed to an HIV gag peptide 'b' *in vitro*, and cultured with autologous CD8⁺ T cells from healthy HIV seronegative donors in the presence of IL-1 and IL-2. The T cells were expanded in culture using the protocol described, *supra*, and shown to be cytotoxic to autologous monocytes pulsed with the same peptide 'b'. This T cell function was antigen-specific, as neither unpulsed monocytes nor monocytes pulsed with another HIV peptide 'a' derived from a distinct region of the gag protein were recognized (FIG. 9). The cytotoxic activity was mediated by MHC class I-restricted CD8⁺ T cells, as evidenced by the ability of monoclonal antibodies against CD8 and HLA class I antigens to

inhibit cytotoxicity. In conclusion, DC pulsed with large polypeptides or small peptides can prime and activate antigen-specific naive CD8⁺ CTL in the
5 absence of CD4⁺ T cells.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention. Indeed, various modifications of the
10 invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

15 All publications cited herein are incorporated by reference in their entirety.

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WHAT IS CLAIMED IS:

1. A method for preparing activated antigen-specific
5 human T cells in vitro comprising co-culturing T
cells with isolated human dendritic cells exposed
to an antigen to activate the T cells to
proliferate or to become cytotoxic in response to
the antigen.
- 10 2. The method of Claim 1 in which the T cells are
CD4⁺.
- 15 3. The method of Claim 1 in which the T cells are
CD8⁺.
4. The method of Claim 1 in which the antigen is a
whole microorganism.
- 20 5. The method of Claim 1 in which the antigen is a
whole virus.
6. The method of Claim 1 in which the antigen is a
polypeptide.
- 25 7. The method of Claim 1 in which the antigen is a
peptide.
8. The method of Claim 1 in which the antigen is a
30 plurality of tumor cells.
9. The method of Claim 1 in which the dendritic
cells are isolated from human peripheral blood.
- 35 10. The method of Claim 1 in which the dendritic
cells are exposed to an antigen by incubation in
culture media.

11. The method of Claim 1 in which the dendritic cells are exposed to an antigen by osmotic lysis of pinosomes.

5

12. A method of activating antigen-specific human T cells in vivo comprising administering into an individual isolated human dendritic cells exposed to an antigen.

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13. A method of identifying an antigen recognizable by T cells comprising co-culturing the T cells with isolated human dendritic cells exposed to the antigen, and measuring T cell proliferation, T cell cytotoxicity or T cell lymphokine production.

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14. A method of isolating human dendritic cells comprising subjecting a mixed population of cells to sequential density gradient centrifugation in the absence of xenogeneic proteins to obtain a final population of $\geq 30\%$ dendritic cells.

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15. The method of Claim 14 in which the dendritic cells are isolated from human peripheral blood.

16. The method of Claim 14 in which the dendritic cells are isolated by Percoll centrifugation followed by Nycodenz centrifugation.

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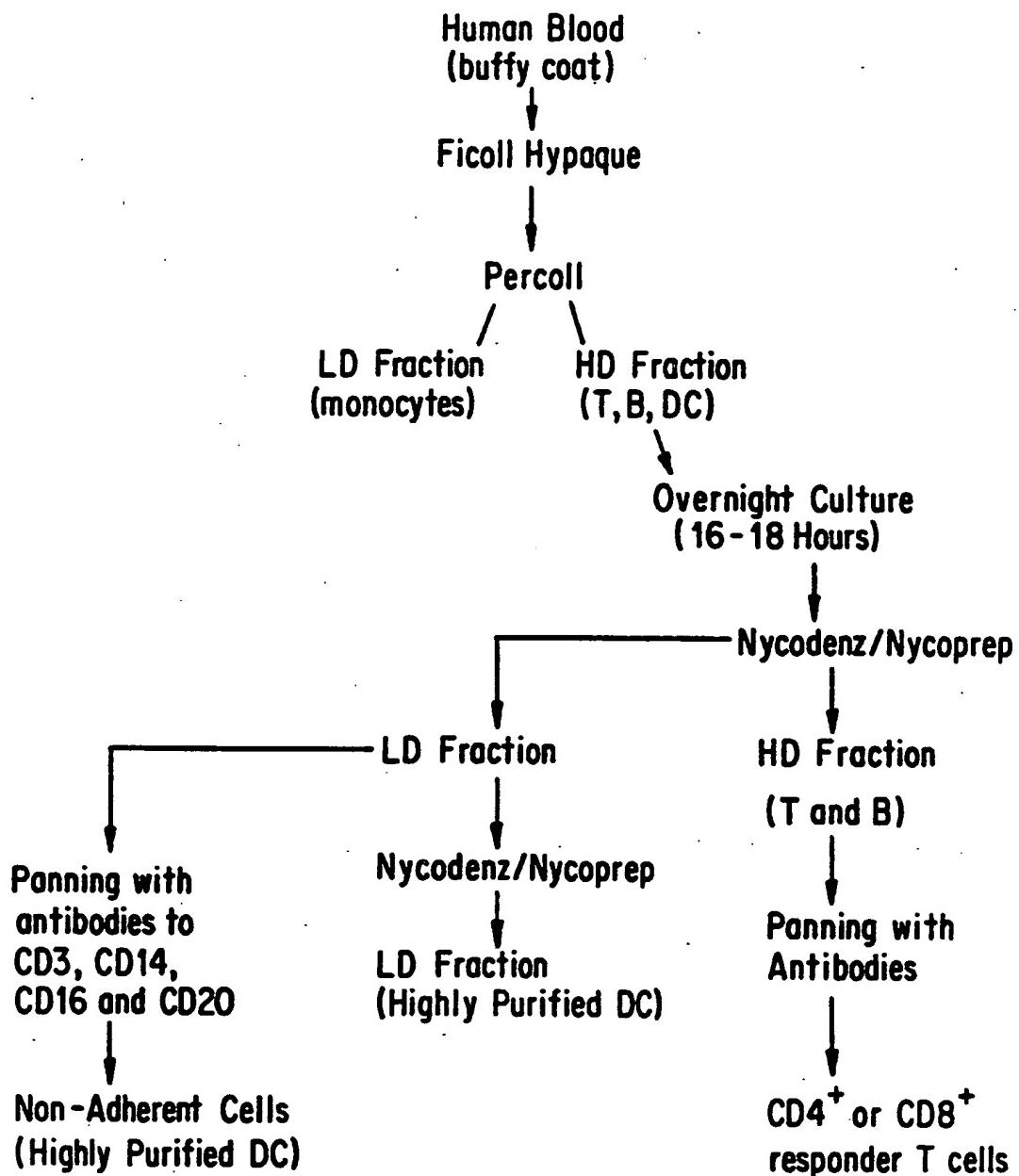
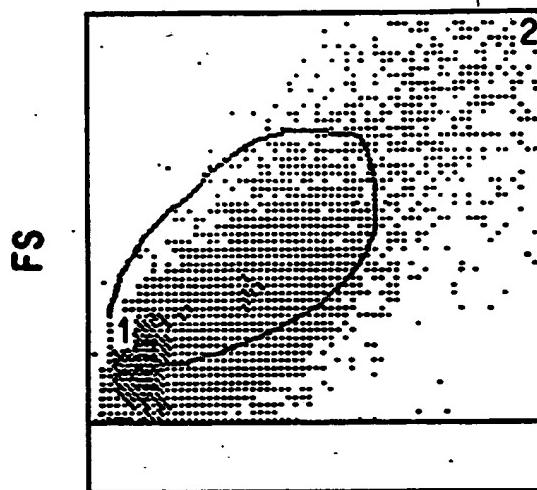


FIG. 1

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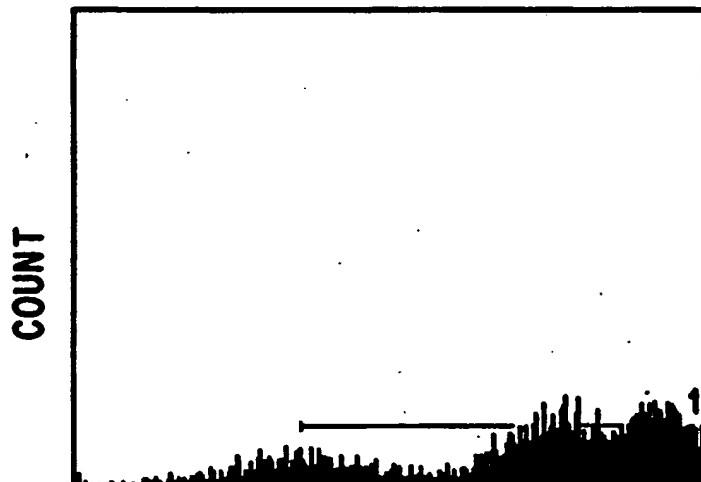
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SS

	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
2 X	0	63	9880	100.0	20.2	16.2	31.6
Y	9	63			25.5	14.1	

FIG. 2A

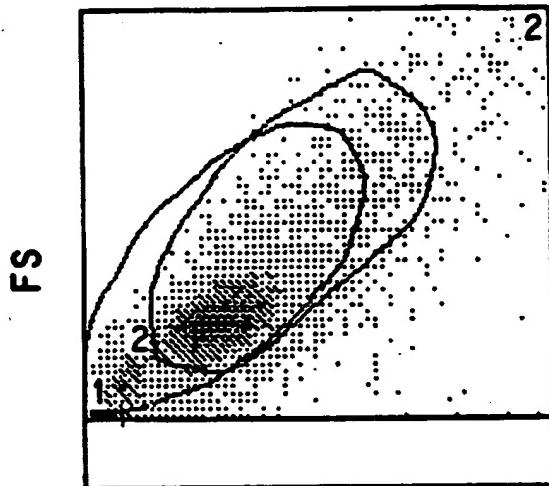


LFL 1

	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	2.705	1023.	4315	86.3	112.5	0.5	

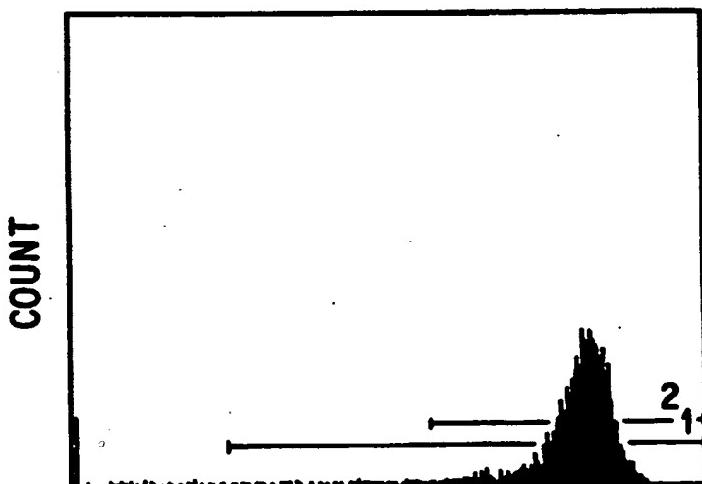
FIG. 2B

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			MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
2 X	0	63	7040		100.0		19.9	12.4	27.5
Y	9	63					23.2	10.6	18.7

FIG. 3A

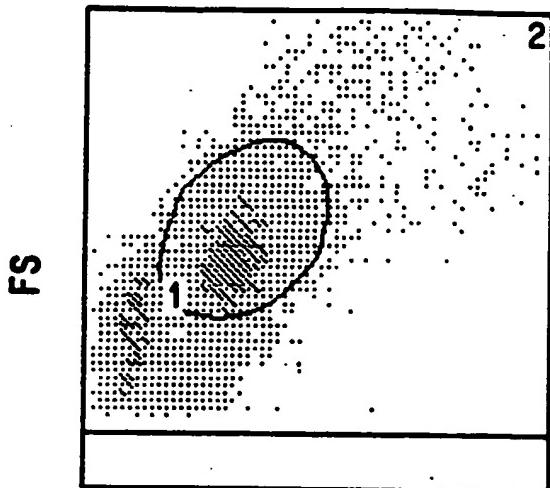


	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1023.	5476	93.1	118.7	0.3	3.95
2	18.88	1023.	5092	86.6	151.1	0.2	3.95

FIG. 3B

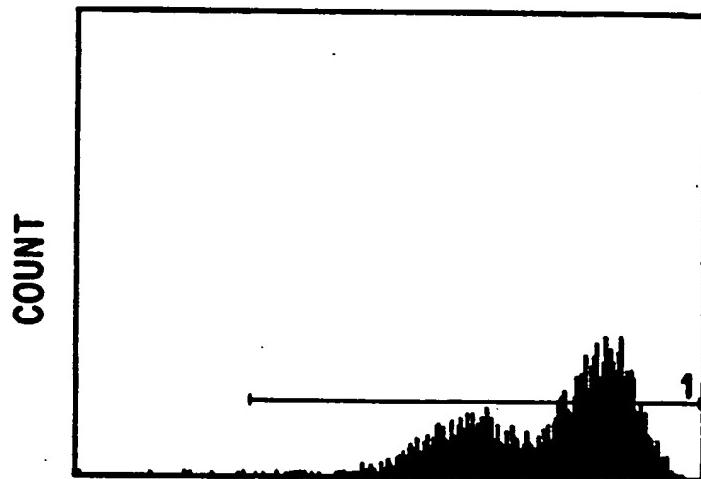
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	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
2 X	0	63	10879	100.0	23.5	16.0	
Y	7	63			33.3	14.7	

FIG. 4A



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.271	1023.	4968	99.4	99.12	0.34	5.28

FIG. 4B

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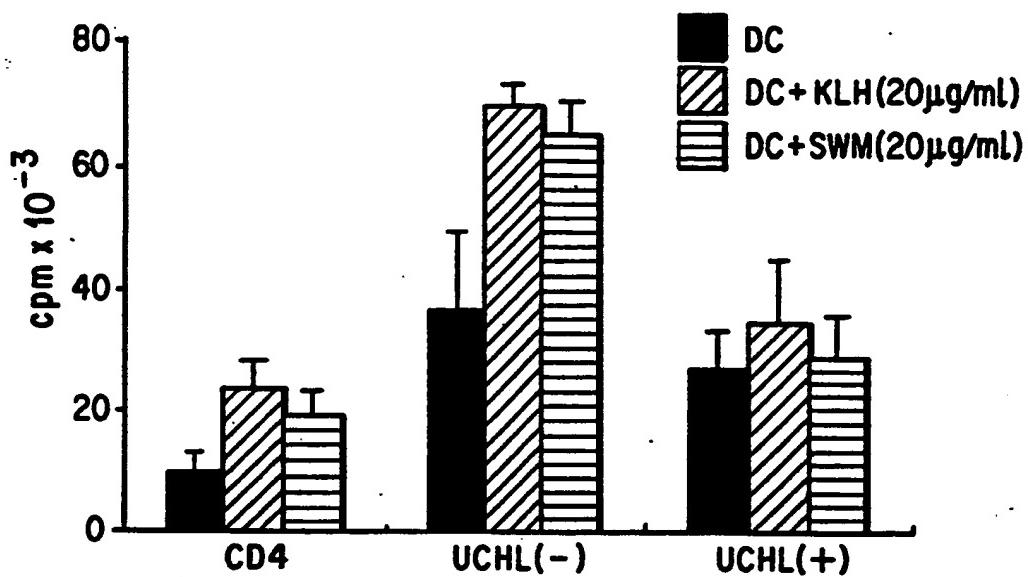


FIG. 5A

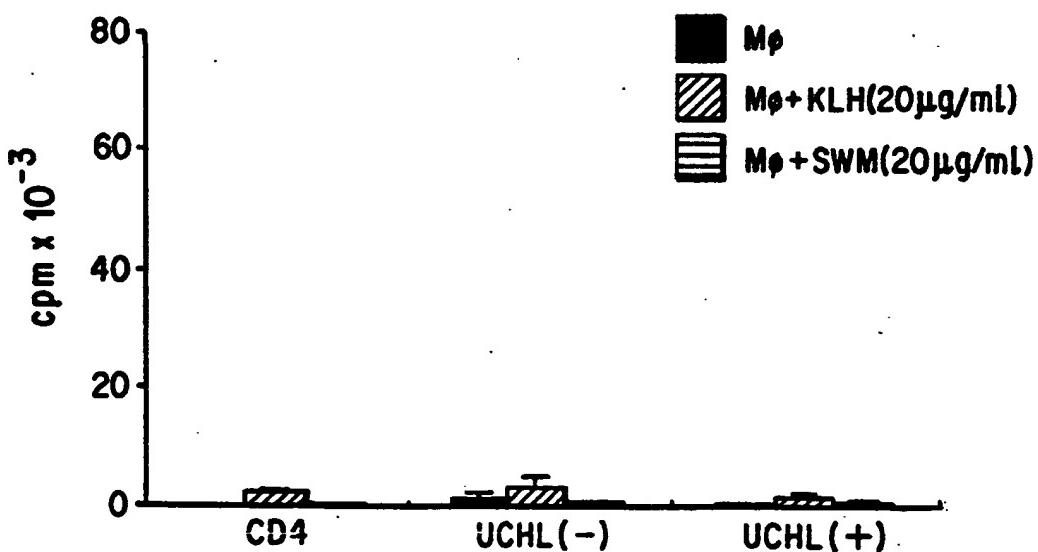


FIG. 5B

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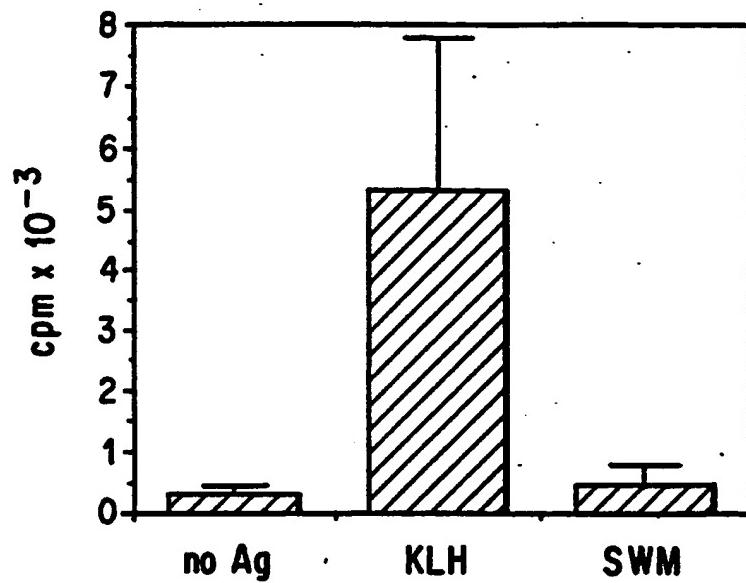


FIG. 6A

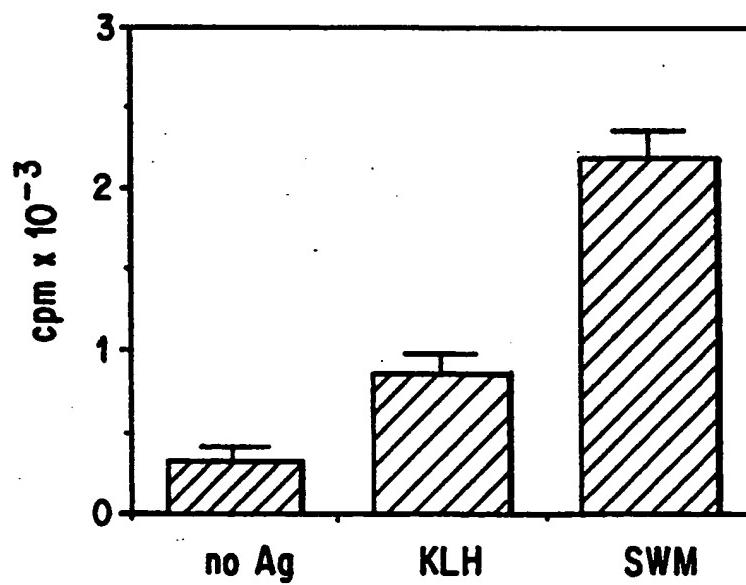


FIG. 6B

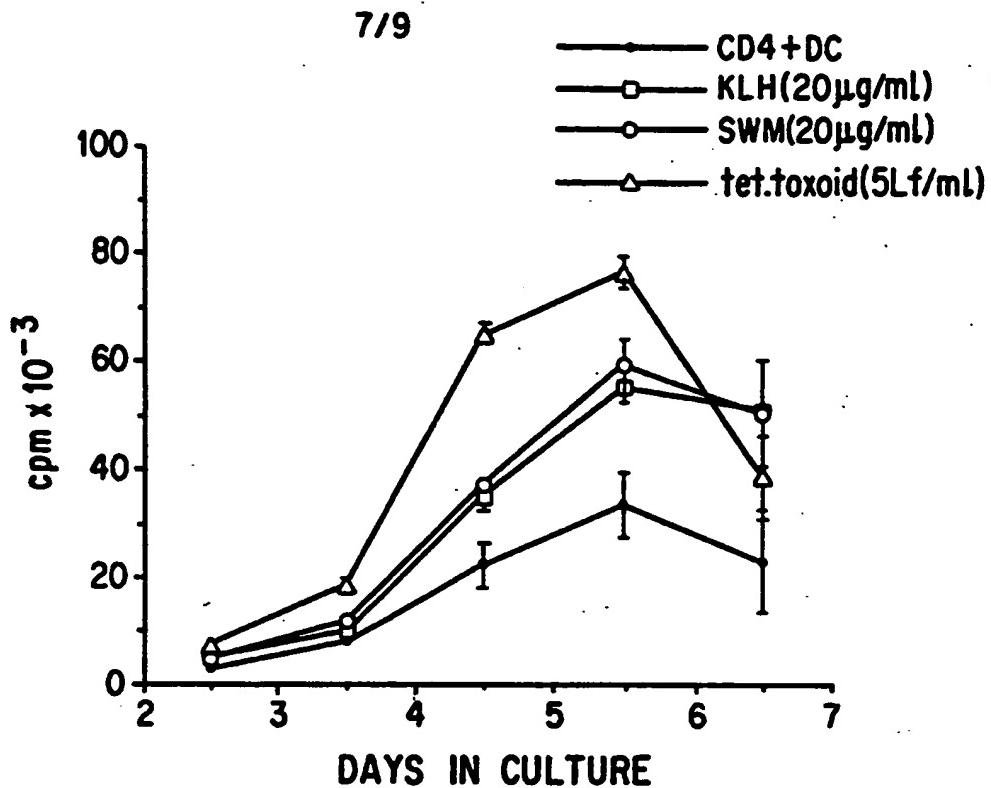
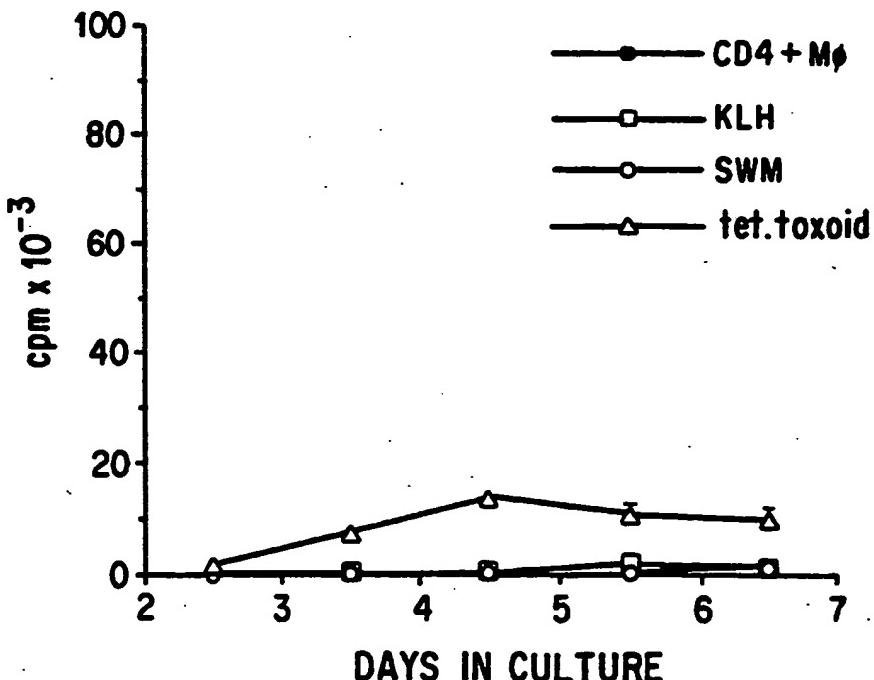


FIG. 7A

FIG. 7B
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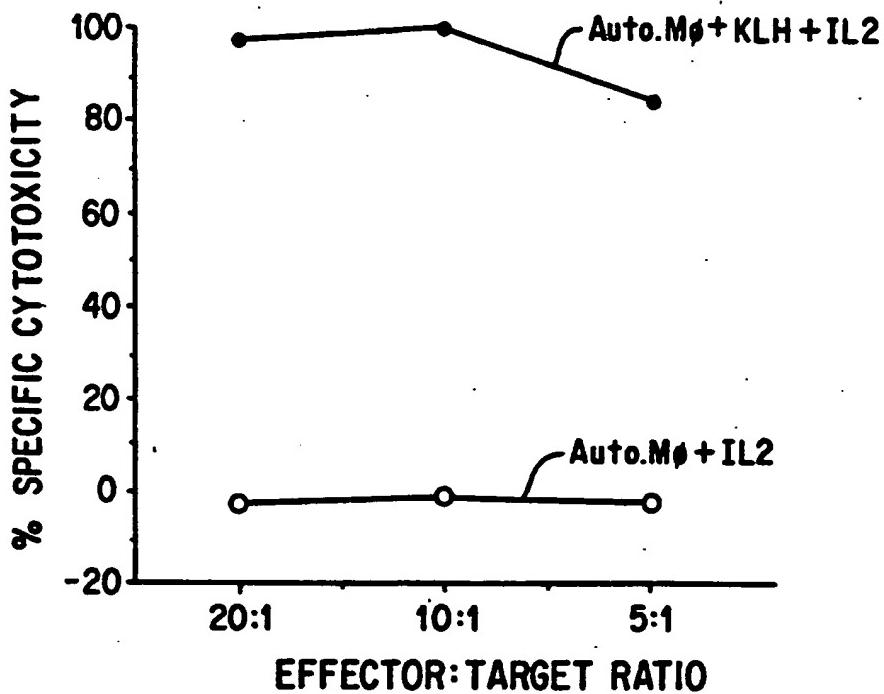


FIG. 8A

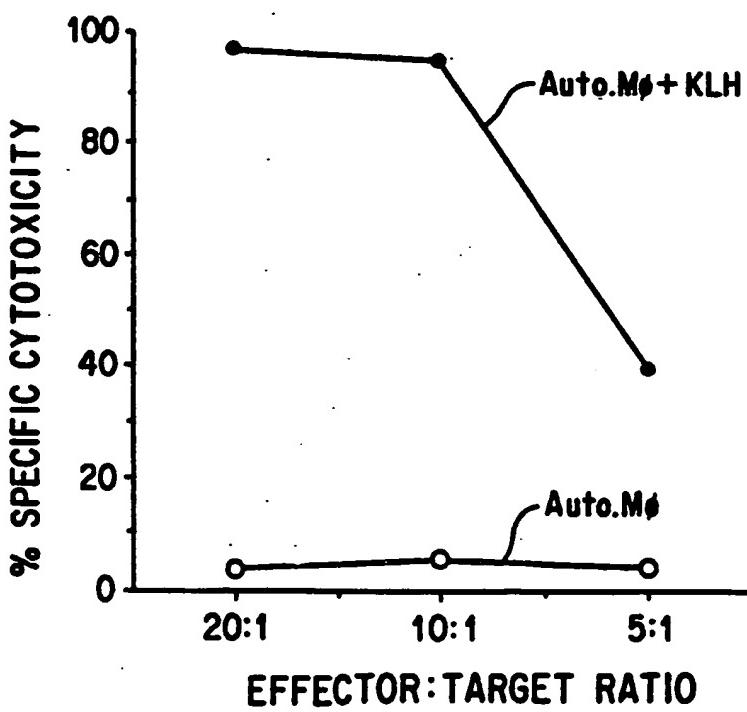


FIG. 8B

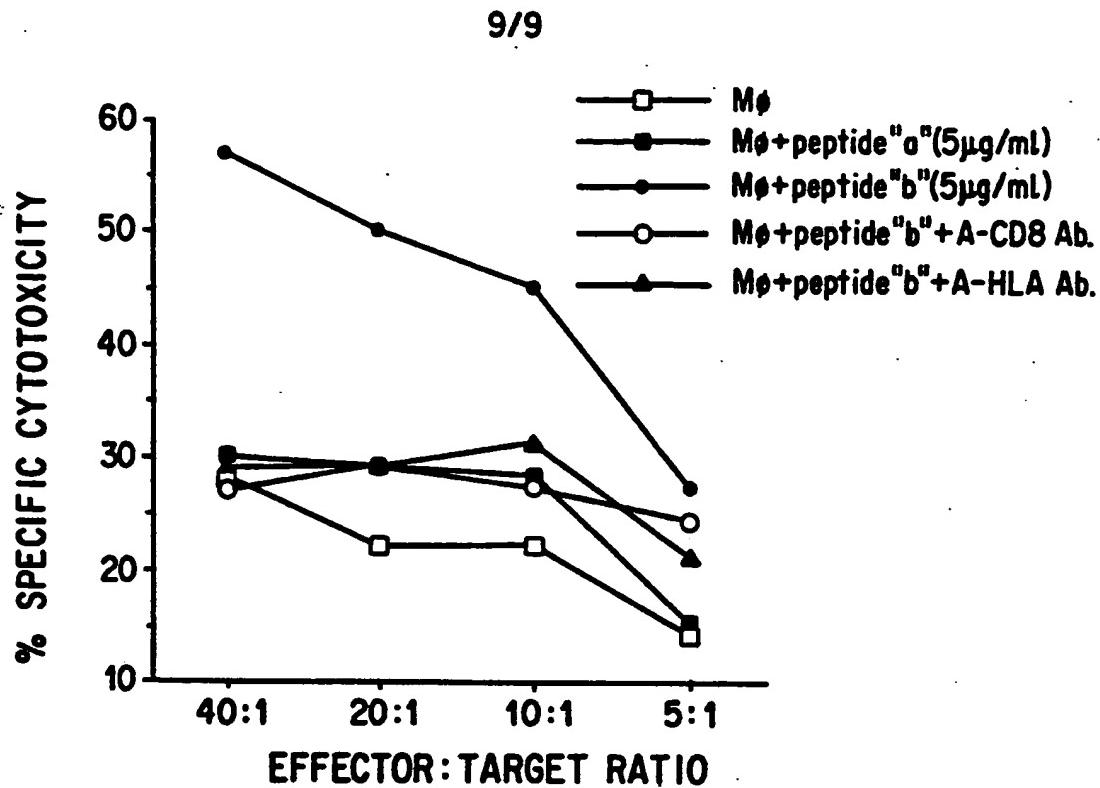


FIG. 9A

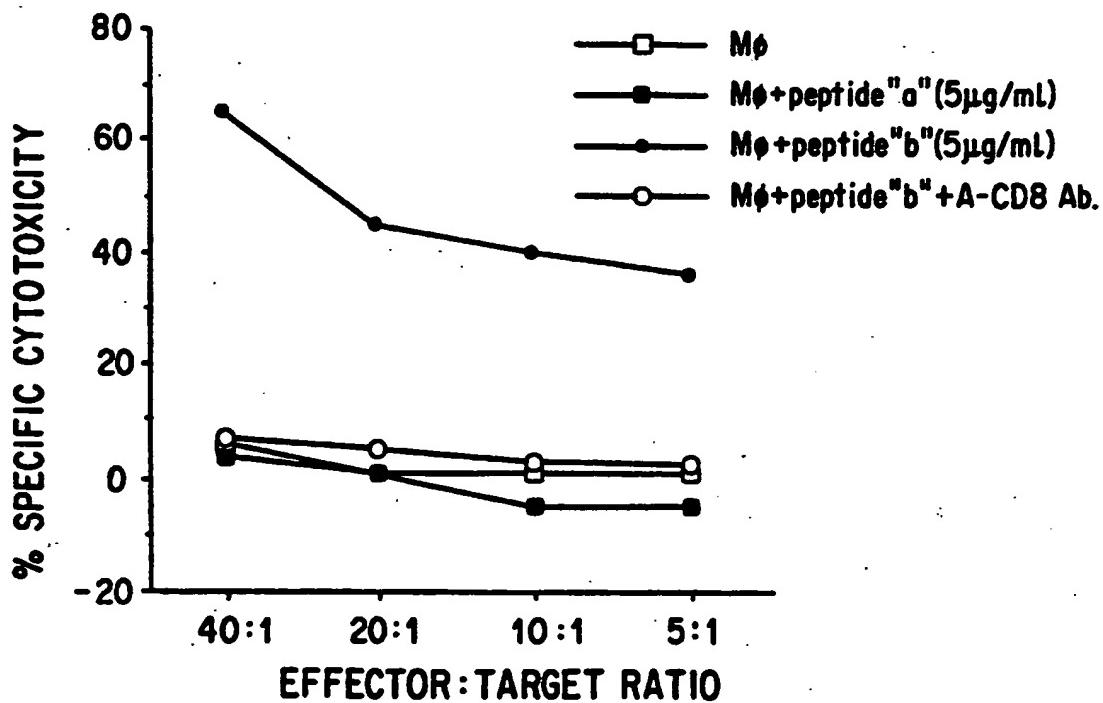


FIG. 9B

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06653

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 35/14, 39/385; C12N 5/08; G01N 33/554, 33/567
 US CL :424/93U; 435/7.24, 2, 240.2; 436/519

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93U; 435/7.24, 2, 240.2; 436/519; 530/810

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG,
 search terms: human, dendritic cell?, antigen(3n)(process? or present? or expos?), nycodenz?, percoll?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF EXPERIMENTAL MEDICINE, Volume 171, issued April 1990, J.W. Young et al, "Dendritic Cells Stimulate Primary Human Cytolytic Lymphocyte Responses in the Absence of CD4+ Helper T Cells", pages 1315-1332, see entire document especially pages 1316 and 1319-25.	1,3,9,10,13-15
Y	CELLULAR IMMUNOLOGY, Volume 111, issued 1988, J.W. Young et al, "Accessory Cell Requirements for the Mixed-Leukocyte Reaction and Polyclonal Mitogens, as Studied with a New Technique for Enriching Blood Dendritic Cells", pages 167-182, see especially pages 168-170.	4-8, 11-12
X		14-15
Y		16

 Further documents are listed in the continuation of Box C. See patent family annex.

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International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF EXPERIMENTAL MEDICINE, Volume 158, issued July 1983, Van Voorhis et al, "Relative Efficacy of Human Monocytes and Dendritic Cells as Accessory Cells for T Cell Replication", pages 174-191, see entire document, especially pages 175-85.	<u>1,2,6,9,10,13</u>
Y		4,5,7,8,12
X	IMMUNOLOGY, Volume 74, Number 3, issued November 1991, S.E. Macatonia et al, "Primary Proliferative and Cytotoxic T-Cell Responses to HIV Induced <i>in vitro</i> by Human Dendritic Cells", pages 399-406, see entire document, especially pages 400-403.	<u>1-7,9-10,13</u>
Y		8,11,12
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Volume 169, issued April 1989, S.E. Macatonia et al, "Primary Stimulation by Dendritic Cells Induces Antiviral Proliferative and Cytotoxic T Cell Responses In Vitro", pages 1255-1264, see entire document, especially pages 1256-7.	1-13
Y	IMMUNOLOGY, Volume 62, issued 1987, J.M. Austyn, "Lymphoid Dendritic Cells", pages 161-170, see entire document, especially pages 161-4.	1-13,16
Y	JOURNAL OF IMMUNOLOGY, Volume 134, Number 3, issued March 1985, P.M. Kaye et al, "Nonphagocytic Dendritic Cells are Effective Accessory Cells for Anti-Mycobacterial Responses In Vitro", pages 1930-1934, see especially page 1932.	1-13
X	JOURNAL OF CLINICAL INVESTIGATION, Volume 85, issued March 1990, S. Markowicz et al, "Granulocyte-Macrophage Colony-Stimulating Factor Promotes Differentiation and Survival of Human Peripheral Blood Dendritic Cells In Vitro", pages 955-961, see pages 955-56.	<u>14-15</u>
Y		16
Y	CELL, Volume 54, issued 09 September 1988, M.W. Moore et al, "Introduction of Soluble Protein into the Class I Pathway of Antigen Processing and Presentation", pages 777-785, see entire document, especially pages 777, 779, and 782-3.	11
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Volume 172, issued August 1990, K. Inaba et al, "Dendritic Cells Pulsed with Protein Antigens In Vitro Can Prime Antigen-Specific, MHC-Restricted T Cells In Situ", pages 631-40, see entire document.	1-13
Y	METHODS IN ENZYMOLOGY, Volume 108, issued 1984, A. Boyum, "Separation of Lymphocytes, Granulocytes, and Monocytes from Human Blood Using Iodinated Density Gradient Media", pages 88-102, see entire document.	16

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